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14. ABSTRACT

Repeat Associated, nonATG (RAN) translation likely plays a key role in ALS disease etiology. Repeat expansions are common, and lead to translation of toxic repeating peptide products. Our project aims to define the mechanisms of RAN translation, and identify inhibitors of the process for therapeutic intervention. Here we report our results from year 1. We have achieved our stated goals to define in vitro and in vivo systems to screen for RAN translation. We have shown that RAN translation can be detected in vitro, and demonstrated the role of the 5' cap in the process. We have performed in vivo screens that have identified co-factors required for RAN translation, and showed that a key ribosomal protein, RpS25, is required for RAN translation in patient-derived induced pluripotent stem cells. This result suggests that RAN translation occurs via a non-traditional pathway that can be selectively inhibited for treatment. Our results in year 1 provide the platform for screening efforts in year 2.

15. SUBJECT TERMS
ALS, RAN translation, drug screens, assay development

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Introduction.

In our research project, we are exploring how GGGGCC repeat expansion in *C9orf72* could cause disease through repeat-associated non-ATG (RAN) translation [3]. This unconventional form of translation occurs in all six reading frames (sense and antisense directions) of the expanded GGGGCC nucleotide repeat, producing polymers of the predicted dipeptides: glycine-alanine (GA), glycine-proline (GP), proline-alanine (PA), glycine-arginine (GR), and proline-arginine (PR). These dipeptide repeat proteins (DPRs) are themselves aggregation-prone and accumulate in the central nervous system of affected *C9orf72* mutation carriers [4-6]. In year 1, we have achieved the goals of our statement of work, and defined systems for high-throughput screening for RAN translation modulation. We have created robust in vitro and in vivo reporters of RNA translation for our screens. Our preliminary work has already identified a key ribosomal protein, RpS25 as a specific modulator of RNA translation, and highlighted a possible cap-dependence to the process. Through the tight collaboration of the Puglisi and Gitler groups, we are poised to understand the mechanisms and inhibition of RAN translation as a therapeutic target in ALS.

Keywords. Repeat-associated non-ATG (RAN) translation, internal ribosome entry site (IRES), ribosome, C9orf72, repeat expansions, RpS25, in vitro translation, single-molecule fluorescence, patient induced pluripotent stem cells (ipSC), genetic screens.

Accomplishments.

Outlined below are the approved Tasks from the SOW for this project, revolving around developing a system and mechanistic understanding for robust screening of compounds that inhibit RAN translation. In year 1, our focus has been on developing the screening platform. We have achieved these goals in both Task 1 and 2 as outlined below:

*Task 1. Mechanism of RAN Translation to develop basis for drug screening (***Puglisi**) (Months 1-24)

Months 1-12	Synthesize and characterize RNA constructs
Months 1-12	Perform bulk translation on mRNAs
Months 6-18	Single-molecule assays for 40S subunit joining and scanning
Months 12-24	Perform single molecule analysis for full initiation and transition to elongation.
Months 12-24	Define factors involved in RAN translation—potential HTS targets
Months 6-18.	Transition bulk and single-molecule assays to HTS (aim 2)

Task 2 Develop high throughput screens for inhibitors of RAN translation (**Gitler/Puglisi**) (Months 18-36)

Months 6-12 Develop, implement and test initial bulk translation high throughput screens for RAN translation (**Puglisi**)
Months 12-18 Perform HTS using screens developed above **(Puglisi**)
Months 18-24 Validate hits from HTS in in vitro assays (**Puglisi)**
Months 18-24 Test hits from HTS in yeast *C9orf72* RAN translation model (**Gitler**)
Months 18-24 Test hits from HTS in HEK293 cell model of RAN translation (**Gitler**)
Months 18-24 Test hits from HTS in iNs from *C9orf72*-mutation carriers and controls (**Gitler**)

*Task 1. Mechanism of RAN Translation to develop basis for drug screening (***Puglisi**)

Our goal is to define the mechanism of G_4C_2 RAN translation by using bulk *in vitro* and single-molecule fluorescence approaches. Requisite to this is the ability to synthesize $(G_4C_2)_n$ mRNA, which is uniquely challenging due to the genomic instability of long repetitive sequences and the physical properties of G-rich nucleic acids.

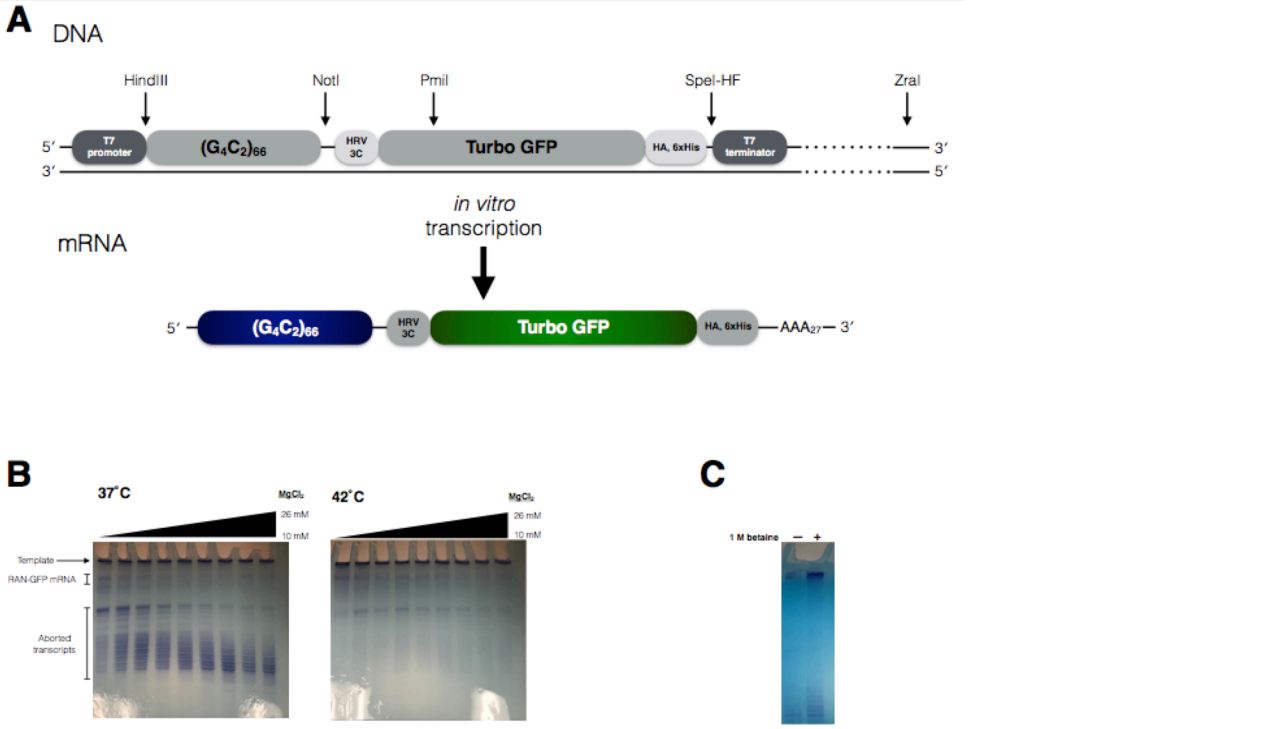

Figure 1: Cloning and synthesis of $(G_4C_2)_{66}$ mRNAs. A) Diagram of DNA template and resulting $(G_4C_2)_{66}$ mRNA B) Gel electrophoresis analysis of trial transcription reactions. C) Gel electrophoresis analysis of $(G_4C_2)_{66}$ mRNA transcribed using optimized transcription conditions. All gels had equal volumes loaded into each well to allow direct comparison of mRNA yield.

Synthesis of mRNAs.

We constructed plasmids containing two or 66 repeats of G_4C_2 ($(G_4C_2)_{66}$) to serve as transcription templates for mRNA synthesis (Fig 1A). The G_4C_2 repeats are flanked by a 5′ T7 polymerase promoter and a 3′ turbo green fluorescent (tGFP) reporter protein coding region in either the 0, +1, or +2 reading frame. Plasmids were designed such that restriction enzymes used to linearize the DNA plasmid for transcription can be selected to yield templates with or without the GFP reporter coding region.

Production of RNAs with repeats is extremely challenging. Initial attempts to transcribe $(G_4C_2)_{66}$ mRNAs from these DNA templates resulted in abortive transcripts of varying lengths and little full-length product, as determined by gel electrophoresis (Fig 1 B). G-rich sequences form stabile quadraplex structures that interfere with enzyme processivity. Therefore, we focused on screening transcription reaction conditions that destabilize nucleic acid secondary structure and found that a combination of high temperature, low magnesium concentration, and the addition of a zwitterionic additive to the reaction yielded full-length $(G_4C_2)_{66}$ mRNAs (Fig 1C). Using these conditions, we have obtained both 5′m^7G-capped and uncapped $(G_4C_2)_{66}$ mRNAs for use *in vitro* translation assays and preliminary single-molecule experiments.

Bulk translation on $(G_4C_2)_{66}$ -tGFP mRNAs. HeLa in vitro translation (IVT) extracts were used to characterize $(G_4C_2)_{66}$ mRNAs. Translation reactions were supplemented with

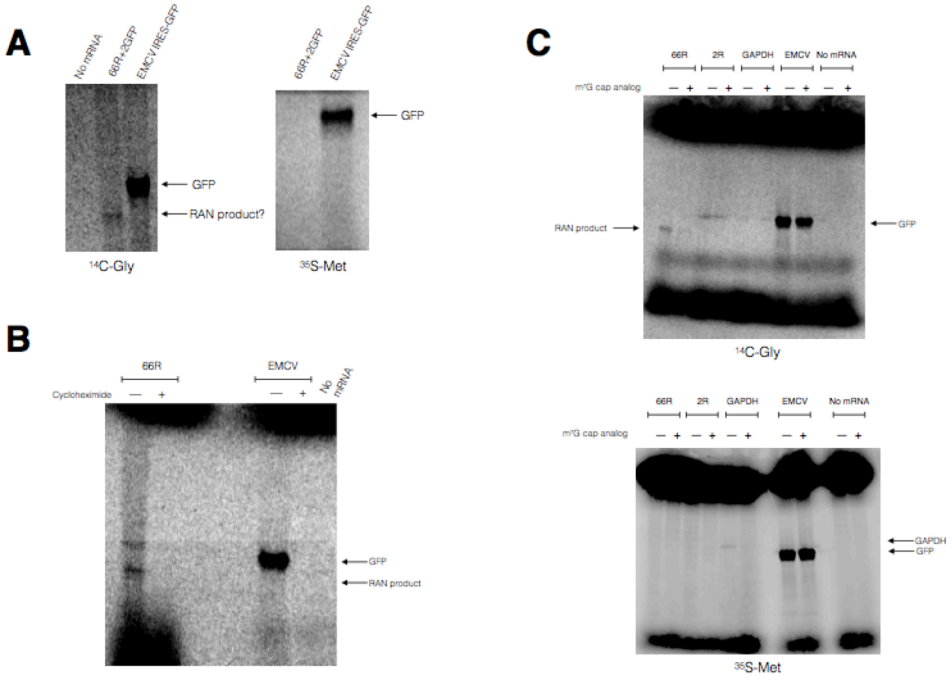

Figure 2: Bulk in vitro translation of $(G_4C_2)_{66}$ mRNA using HeLa extractions. Phosphor images of IVT assays A) Detection of +1 frame RAN translation products in the context of the +2 frame $(G_4C_2)_{66}$ mRNA via ^{14}C-Gly. The +1 frame band is not detected by ^{35}S because it lacks a Met residue. B) Translation of +1 frame mRNA and EMCV IRES driven translation are inhibited by cycloheximide. C) Inhibition of cap-dependent mRNA translation by cap analog

either ^{35}S-Met or ^{14}C-Gly so that translation products can be detected as radioactive

bands on a SDS-PAGE gel. Our first observation was that the translation efficiency of $(G_4C_2)_{66}$ -tGFP mRNAs is low compared to EMCV IRES-driven tGFP translation (Fig 2).

Our initial IVT results suggest $(G_4C_2)_{66}$ mRNAs are more readily translated in the +1 frame. Translation of the $(G_4C_2)_{66}$ mRNA with tGFP in the +2 reading frame results in a band consistent with translation initiation in the +1 frame whereas the signal from the +2 frame translation product is just above background (Fig 2). Translation of the $(G_4C_2)_{66}$ +1 frame is inhibited by the addition of cap analog to the translation reaction indicating $(G_4C_2)_{66}$ translation in cap-dependent. We will base our initial design for HTS using the +1 reporter system.

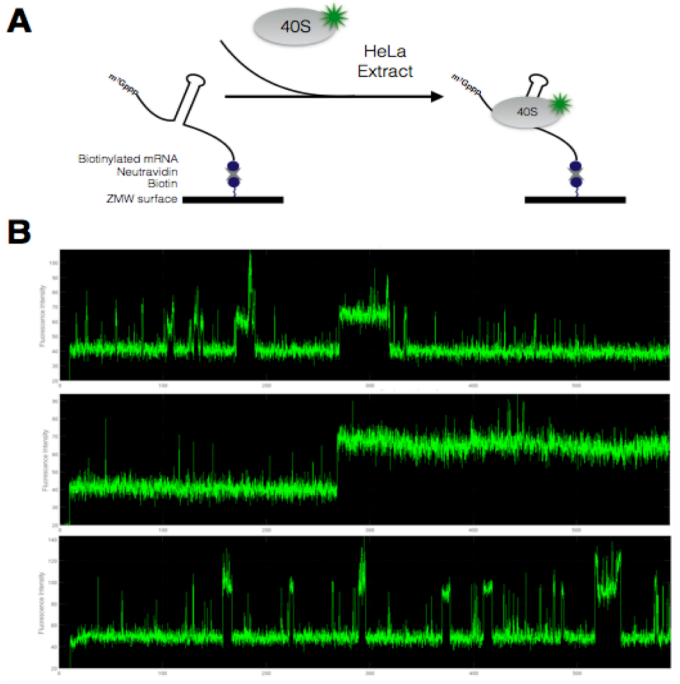

Figure 3: Single-molecule fluorescence detection of 40S ribosome recruitment to $(G_4C_2)_{66}$ mRNA. A) Schematic of the experimental set up. B) Representative traces in which pulses of green fluorescence indicate 40S subunit binding. The middle panel is an example of a stable binding event, whereas the top and bottom traces a sampling events.

Single-molecule fluorescence assays for 40S subunit joining. Sm fluorescence assays of G_4C_2 RAN translation are being developed in parallel with bulk translation assays. In our pilot study, we have directly observed $(G_4C_2)_{66}$ mRNA-mediated recruitment of the 40S ribosomal subunit by delivering HeLa extract dosed with fluorescently-labeled, human 40S ribosomal subunits to surface-immobilized $(G_4C_2)_{66}$ mRNA (Fig 3).

Task 2 Develop high throughput screens for inhibitors of RAN translation (**Gitler/Puglisi**) (Months 18-36)

Test hits from HTS in yeast *C9orf72* RAN translation model (**Gitler**)
Months 18-24 Test hits from HTS in HEK293 cell model of RAN translation (**Gitler**)
Months 18-24 Test hits from HTS in iNs from *C9orf72*-mutation carriers and controls (**Gitler**)

Thorough and reproducible assays in both yeast and mammalian systems were required to validate hits from the HTS. One of the primary goals was to screen the yeast Mat a Deletion Library of nearly 5,000 nonessential genes for modifiers of RAN translation levels. Several potential reporter systems were tested including GFP, Firefly luciferase, and lacZ as readouts of RAN translation in a high-throughput screen. These reporters either did not yield reliably consistent measurements or were not amenable to high-throughput screening due to low background-to-signal ratios (data not shown). Therefore, an ELISA assay against the glycine-proline RAN dipeptide product was also tested for amenability to high-throughput analysis of yeast lysates (Fig. 4). Reproducible results were achieved using the ELISA assay when testing an initial set of yeast deletions for modulation of RAN-specific GP production in comparison to modulation of cap-dependent YFP production. Further, another 200 yeast deletion strains were tested in biological replicate as mini-screen. Out of these 200, 15 strains were identified for further validation to 1) determine if the deletion influences C9 transcript levels and 2) determine the effect of the deletion on ATG-YFP expression. Figure 4D illustrates the effect several deletions which include *mdm38*Δ where RAN translation levels (polyGP) increased, but the levels of YFP and C9 RNA remained unchanged. These deletions are now being tested in mammalian systems to determine if the effect on RAN is conserved.

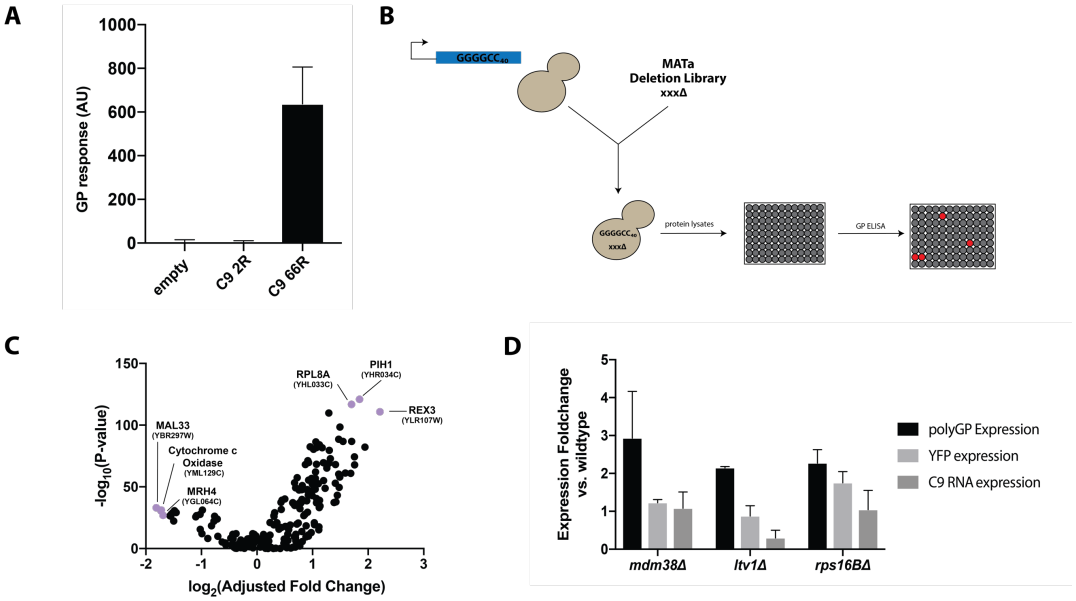

Figure 4: GP ELISA-based screen for RAN translation in yeast. A) ELISA assay to detect RAN translation products from yeast lysates harboring 2 or 66 GGGGCC repeats. The ELISA assay (performed by the Petrucelli laboratory at Mayo Clinic Jacksonville) detects the production of one RAN translation product (GP) robustly and demonstrates the ability of yeast cells to initiate RAN translation. **B)** Schematic of the mini-screen conducted with 200 deletions from the MATa Deletion Library collection of yeast haploid deletion strains. **C)** Volcano plot illustrating fold-change of GP levels in each yeast deletion

strain compared to wildtype yeast. **D)** Three selected deletions shown to illustrate follow-up studies to determine effect of each deletion of YFP levels (effect on general translation) and on C9 RNA levels. The *mdm38Δ* strain illustrates an example of a yeast strain where RAN translation increased but YFP and C9 RNA levels remained unchanged compared to wildtype yeast.

Where the GFP reporter in a yeast system was not amenable to high-throughput screening due to the high autofluorescence yeast display, RAN GFP reporters were especially robust in a mammalian expression system. EGFP was inserted after 2 or 66 G_4C_2 repeats in each potential RAN reading frame to generate 6 unique vectors. Consistent with previous reporters and the length-dependent hypothesis, RAN GFP products were only detected when GFP was inserted after 66 repeats but not 2R (Figure 5). The mammalian RAN GFP reporter will allow for quick screening of HTS hits utilizing FACS or more traditional biochemical approaches such as Western blot. Furthermore, the system could allow for HTS via CRISPR or drug screens to complement the in vitro screens using high-throughput microscopy or FACS.

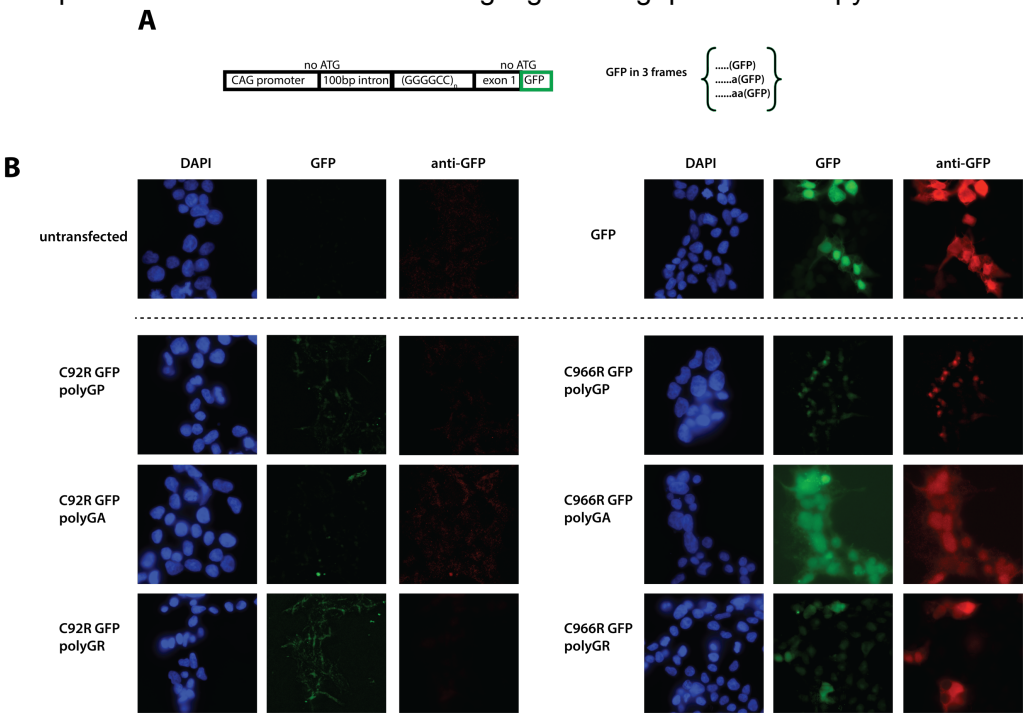

Figure 5: Mammalian G_4C_2-GFP reporters are robust readouts of RAN translation. A) Schematic of G_4C_2-GFP vectors. GFP is inserted in each frame after 2 or 66R. **B)** GFP is only observed when appended after 66R (right) but not 2R (left).

The repeat-harboring RNAs of RAN translated genes are known to form unique and complex secondary structures (cite that one paper) and have been hypothesized to potentially utilize an IRES-mediated initiation. Thus, we tested several ribosomal subunits with previously described roles in IRES-mediated translation. Of these, the deletion of RPS25A reduced the levels of RAN product poly-GP in yeast (Figure 6A). [filler about RPS25 here?] This effect was further investigated in mammalian Hap1 cell lines with a CRISPR-mediated knockout of RPS25. Similar to the effect in yeast, RPS25KO reduced levels of RAN poly-GP by 50%. Strikingly, the reduction of poly-GA product via western blot was even more dramatic to ~7.8% of wildtype poly-GA levels illustrating the inhibitory effect of RPS25KO on at least two frames of RAN translation (Figure 3A-D). Puromycin-incorporation assays of RPS25KO versus wildtype cells confirm previous observations that RPS25KO does not have an effect on global translation

(Figure 6F, Citations). Further, polysome profiles of actively translated mRNAs and growth curve analysis of RPS25KO and wildtype cells do not indicate differences (data not shown).

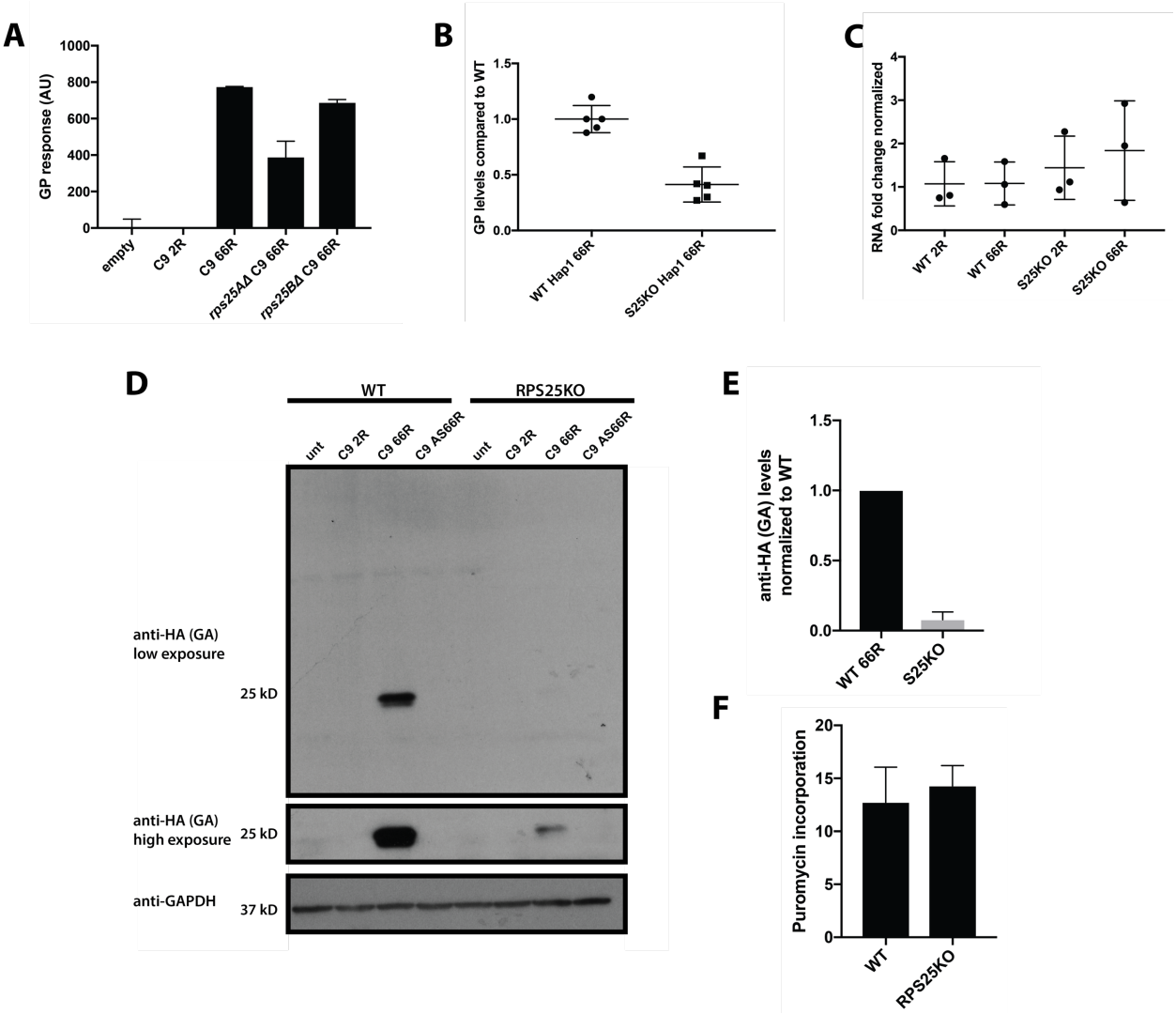

Figure 6: RPS25KO reduces levels of RAN translation products. A) *RPS25AΔ* reduces level of poly-GP in yeast expressing 66 G_4C_2 repeats compared to a wildtype strain by GP ELISA. **B)** The effect of RPS25 reduction on RAN translation is conserved from yeast to mammalian cell lines. Hap1 mammalian cell lines were transiently transfected with 66 G_4C_2 repeats and subsequently analyzed via GP ELISA. RPS25KO cell lines express reduced levels of poly-GP consistent with yeast data. **C)** qRT-PCR data illustrating that G_4C_2 RNA is not reduced as a result of RPS25KO. **D)** Poly-GA can be detected by an HA-epitope tag inserted into the putative GA-frame. Poly-GA is substantially decreased in RPS25KO cell lines. **E)** Quantification of panel D. **F)** Cells treated with puromycin are harvested and immunoblotted for puromycin incorporation into nascent peptides with anti-puro immunoblot. Quantification illustrates that RPS25KO cells incorporate puromycin at equivalent levels, supporting existing evidence that RPS25KO does not have an effect on global translation.

We then tested whether RPS25 reduction would decrease levels of RAN translation products in patient-derived iPSCs where the repeat expansion occurs in an endogenous context. Reduction of RPS25 levels using shRNA reduced the levels of poly-GP to ~25% (Figure 7A,B). Importantly, the RPS25 reduction did not influence transcript levels (Figure 6C) or ability to form RNA foci (Figure 7C), indicating RPS25's role at the level of translation and not influencing

repeat RNA dynamics. These results indicate that RPS25 is a clear modifier of RAN translation and underlie the strength of the approaches used to characterize more RAN translation machinery.

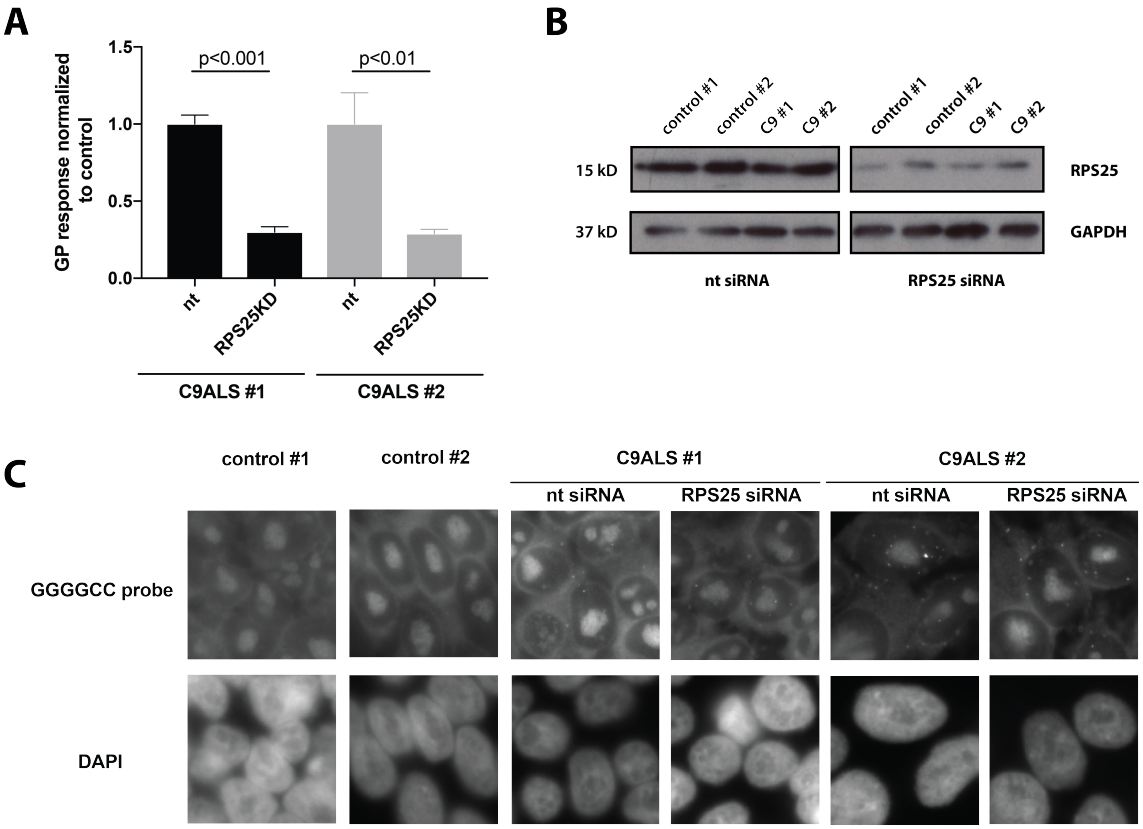

Figure 7: RPS25KD in C9ALS patient derived iPSCs reduces levels of poly-GP. A) GP ELISA assay on two C9ALS patient iPSCs treated with non-targeting (nt) or RPS25-targeting (RPS25KD) siRNAs. KD of RPS25 reduces GP levels to about 25% of the levels expressed in control-treated equivalents. Note that the levels of GP are much higher in patient #1 versus #2 and that age-matched control cell lines do not express any detectable poly-GP (data not shown). **B)** Immunoblot indicating RPS25KD in non-targeting versus RPS25-targeting siRNAs. **C)** RNA FISH on patient iPSCs using G_4C_2-RNA hybridizing probe. RNA foci are not detected in control cell lines. RNA foci dynamics are not disturbed in RPS25KD versus non-targeting siRNA treatment.

Impact. The impact of our work during the past 12 months is potentially strong. We have, for the first time, defined in vitro and in vivo systems to study RAN translation, its mechanism and potential inhibitors. We have identified factors in patient cells that modulate RAN translation. RpS25 is known to be important for viral IRES mediated translation but not host, cap-dependent translation. This is the first hint that RAN translation proceeds by a novel, and targetable pathway.

Changes/Problems. Our first year hued closely to plan, and we are satisfied with the creation of both in vitro and in vivo systems. We are now focusing on transforming these

systems to a platform to give high-confidence screening results, and subsequent validation.

Products. We have produced a range of expression systems, and cell lines to explore RAN translation, that will be made available to the community.

Participants and other Collaborating Organizations

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